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ORIGINAL

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PROVISIONAL SPECIFICATION

Invention Title: "Cryptosporidium Propagation Systems"

The invention is described in the following statement:

"Cryptosporidium Propagation Systems"

Field of the Invention

The present invention relates to propagation systems for the intestinal protozoan, *Cryptosporidium.* It relates to host-cell free maintenance and/or propagation media capable of use in the methods for propagation and amplification of *Cryptosporidium.* It also relates to the use of *Cryptosporidium* so prepared by the propagation systems or media of the invention in the preparation of vaccines and therapeutics suitable for use in the treatment and prevention of human and animal diseases caused by *Cryptosporidium.* This method also will help in developing a specific diagnostic test for the detection of *Cryptosporidium*, particularly for the detection of *Cryptosporidium* in water samples.

Background Art

Intestinal protozoa cause a variety of clinical and economically important diseases in human and animals. Examples of known pathogenic intestinal protozoa include *Giardia*, trichomonads, *Histomonas*, *Spironucleus*, *Entamoeba*, Coccidia, *Sarocystis* and *Cryptosporidium.*

Cryptosporidium is an Apicomplexan protozoan parasite that invades the intestinal epithelial cells of humans and various mammalian hosts, domesticated farm animals and poultry. In humans, the parasite infects the microvillus border of the intestinal epithelium, causing acute, self-limiting diarrhoea in immunocompetent individuals, and a chronic, life-threatening disease in immunocompromised patients. *C. parvum* demonstrates broad mammalian host specificity, infecting humans through direct human contact and via zoonotic transmission and has become a leading cause of diarrhoea in calves. *C. baileyi* and *C. muris* have greater specificity for chickens and mice, respectively. [Laurent, F. *et al* (1990) Microbes and Infection 2:141-148; Gasser, R.B. and O'Donoghue, P. (1999) Int J Parasitol 29:1379-1413].

At least two species of *Cryptosporidium* infect cattle. *C. parvum* is characterized by small-type oocysts (5.0 x 4.5 μm) and primarily infects the intestine of young calves resulting in considerable economic losses in the cattle industry and water-

borne outbreaks of diarrheal disease in human populations [O'Donoghue, P.J. (1995) Int J Parasitol 25:139-95]. *C. andersoni* is a recently renamed species characterized by larger oocysts 97.4 x 5.6 mm) that infects the abomasum (fourth division of the stomach in ruminant animals) of cattle [Lindsay, D.S. *et al* (2000) J Eukaryot Microbiol 47:91-95]. To date, no effective treatment for cryptosporidiosis is available.

Cryptosporidium life cycle and propagation

Cryptosporidium oocysts are transmitted by the fecal-oral route, and can be transmitted through contaminated water supplies and public swimming pools in endemic regions, transmits. Following ingestion by a suitable host, *Cryptosporidium* oocysts excyst in the presence of host bile salts and pancreatic enzymes. The resulting sporozoites infect intestinal epithelial cells, and differentiate into trophozoites. The trophozoites multiply asexually to produce type I schizonts containing about 6-8 merozoites. These merozoites can invade additional cells upon rupture of the schizonts. Merozoites may continue to develop into type I schizonts or form type II schizonts, which further differentiate into either male microgamonts or female macrogamonts. Male microgamonts release microgametes that fertilize the female macrogamont, resulting in an oocyst. Thick-walled oocysts pass through the digestive tract of the host while thin-walled oocysts likely reinfect the host [Laurent, F. *et al* (1999) Microbes Infect 2:141-148; Gasser, R.B. and O'Donoghue, P. (1999) Int J Parasitol 29:1379-1413].

Oocysts and sporozoites can be obtained from infected animals (e.g., calves) in large quantities; however, the handling and maintenance of infected animals constitute a risk of infection to humans. In addition, obtaining parasites from infected animals presents difficulties in terms of standardization of assays and experimental reproducibility.

The ability to propagate *Cryptosporidium in vitro* was first achieved in the endodermal cells of the chorioallantoic membrane (CAM) of chicken embryos [Current, W.L. and Long, P.L. (1983) J Infect Diseases 148:1108]. Numerous subsequent reports of the cultivation of the parasite in different cell lines followed,

with varying degrees of success in terms of the development of *Cryptosporidium* life cycle stages [Hijjawi, N.S. (2003) In: "*Cryptosporidium* from molecules to disease", R.C.A. Thompson, A. Armson, and U. Ryan (Eds.). Elsevier, Amsterdam, The Netherlands, Ch 31:233-253]. Although major improvements in the *in vitro* culture of *Cryptosporidium* have occurred in recent years, continuous culture and efficient life cycle completion (oocyst production) have only recently been achieved *in vitro*, with long term maintenance of the life cycle of three species of *Cryptosporidium* (*C. parvum*, *C. hominis* and *C. andersoni*) now possible [Hijjawi, N.S. *et al* (2002) Int J Parasitol 32:1719-1726; Hijjawi, N.S. *et al* (2001) Int J Parasitol 31:1048-1055].

It is well recognised that successful *Cryptosporidium* cell culture systems have aided many aspects of *Cryptosporidium* research. The ability to culture *Cryptosporidium in vitro* has allowed the screening of antimicrobial compounds, enhanced studies on the developmental biology and life cycle of the parasite as well as supporting the needs of the water industry in terms of detection and viability screening.

However, a number of important problems remain with the current culture methods, such as the need for different host cell lines and problems with overgrowth and aging of host cells, which can prevent perpetuation of the *Cryptosporidium* life cycle *in vitro*.

The present invention addresses a need in the art for improved *Cryptosporidium* propagation systems capable of at least ameliorating one or more of the problems attendant with the prior art.

Summary of the Invention

The invention is drawn to improved methods for the culture of *Cryptosporidium* that are host-cell free and therefore offer a range of advantages over presently existing culture methods. Culture systems described herein are much easier to use than previous systems which required the presence of different host cell lines and potentially overcome problems of overgrowth and aging of host cells which prevent perpetuation of the *Cryptosporidium* life cycle *in vitro*. Furthermore, a

host cell free *Cryptosporidium* culture system will assist in future vaccine development against cryptosporidiosis, especially in terms of scaling up of the culture of parasite stages, harvesting of parasite stages free of host contamination, and the simplification of drug-screening protocols.

According to one aspect, the invention provides a method for culturing *Cryptosporidium* comprising the steps of:

(a) Introducing a stage in the life cycle of *Cryptosporidium* into culture media selected from a maintenance medium or a biphasic medium in the absence of host cells; and

(b) Culturing the *Cryptosporidium*.

In addition to improved culture systems the invention is drawn to a biphasic culture medium that has been found suitable for host-cell free growth of *Cryptosporidium*.

According to this second aspect, the invention provides a biphasic medium for the cultivation of *Cryptosporidium*, comprising a semi-solid medium base phase and a liquid medium overlay.

The invention also includes oocysts produced by the new propagation method and compositions thereof. In a preferred embodiment, the invention includes a composition comprising oocysts produced by a culture system described herein and a pharmaceutical carrier.

The invention further encompasses the use of *Cryptosporidium* produced by the systems described herein for inducing antibodies in an animal, especially antibodies providing protection against cryptosporidiosis. Methods for producing intestinal protozoan vaccines are described, for example, by Olson in U.S. Patent Nos. 5,512,288 and 6,153,191, which are hereby incorporated by reference. The extracellular forms of *Cryptosporidium* of the present invention will be particularly useful in producing a vaccine that will elicit an immune response with therapeutic benefit in animals and humans. The invention also includes a vaccine composition comprising oocysts produced by a culture system described herein.

In a preferred embodiment, the composition comprises a suitable pharmaceutical carrier, which may be an adjuvant.

The invention also encompasses a method of producing antibodies to *Cryptosporidium* in an animal comprising administering to the animal an antibody-producing amount of the oocysts produced by a culture system described herein. In a preferred embodiment, the antibodies confer a therapeutic benefit (for example they confer protective immunity) to the animal suffering from or susceptible to a *Cryptosporidium* infection.

The invention further encompasses the use of *Cryptosporidium* produced by the new culture methodology for preparing a therapeutic according to methods known in the art, such as those described in the Olson patents. In one embodiment of the invention, the oocysts used in the therapeutic are cryopreserved in a cryopreservation solution comprising 10% DMSO and 90% FBS.

In a further embodiment, the oocysts are recovered by centrifugation of the supernatant from the culture system described herein.

Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following illustrative drawings.

Brief Description of the Drawings

Figure 1: Sporozoites released from *Cryptosporidium parvum* oocysts. **a.** Sporozoites transformed to trophozoites, which are circular to oval in shape. **b&c.** Note how trophozoites aggregate together after their release from oocysts. **d.** Note most of oocysts detected after 24 hours were empty. 24 hrs culture of *Cryptosporidium parvum* oocysts in RPMI-1640 monophasic maintenance medium. Scale bar = 5μm.

Figure 2: Meront I formed after the fusion of trophozoites released from *Cryptosporidium parvum* oocysts. Note the size of these meronts depend on the number of trophozoites clumped together. 72 hrs culture of *Cryptosporidium parvum* oocysts in RPMI–1640 biphasic maintenance medium. Scale bar = 5μm.

Figure 3: **a**. Early Meront II. **b**. Meront II appeared as rosette with merozoites in the process of formation. **c**. Meront II releasing merozoites. **d&e**. Free merozoites released from Meront II, note some of them are spindle-shaped with pointed ends and others are circular. 8-day-old culture of *Cryptosporidium parvum* oocysts in RPMI-1640 monophasic maintenance medium. Scale bar = 5μm

Figure 4: Merozoites after 8 days of culture in RPMI-1640 biphasic maintenance medium. **a**. Note the productivity of this system where you can see a large number of merozoites formed; also note the presence of two morphologically different types of merozoites some spindle shaped with pointed ends and others circular. Scale bar = 5μm.

Figure 5: Sexual stages detected in *Cryptosporidium parvum* grown in RPMI-1640 biphasic maintenance medium for 6-7 days. **a**. Microgamonts with microgametes, which eventually bud off (b) from the surface. **b**. Early microgamont with developing microgametes where their nuclei are clearly shown. **c**. Late macrogamont with microgametes adhered to the surface. **d**. Microgametes still clumped together upon their release from microgamonts. **e**. Free fully developed microgametes, note the nucleus filling most of the cytoplasm. **f**. Fully developed macrogamont with peripheral nucleus. **g**. Fertilization process where you can see macro and microgametes fusing together (Mi & Ma) and a microgamete still adhering to the surface. **h**. Fertilization in process as macro and macrogamont (Ma & Mi) pair together. **i**. Free zygote (unsporulated oocyst) with big central nucleus. Scale bar = 5μm.

Figure 6: *Cryptosporidium parvum* sporulated oocysts after 46 days of culturing in RPMI-1640 biphasic maintenance medium with the continuous release of sporozoites. Scale bar = 5μm

Figure 7: Extracellular stages detected in biphasic culture after 8 days in RPMI-1640 biphasic maintenance medium. Scale bar = 5μm.

Figure 8: *Cryptosporidium parvum* life cycle in host cell free medium.

Figure 9. Diagrammatic illustration of the life cycle of *Cryptosporidium parvum* in host cell free medium.

Detailed Description of the Invention

General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

As used herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the work "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as

commonly understood to one of ordinary skill in the art to which the invention belongs.

Description of the Preferred Embodiment

A requirement currently exists to provide a culture system suitable for the propagation of protozoa such as *Cryptosporidium*.

According to one aspect, the invention provides a method for culturing *Cryptosporidium* comprising the steps of:

(a) Introducing a stage in the life cycle of *Cryptosporidium* into culture media selected from a maintenance medium or a biphasic medium in the absence of host cells; and

(b) Culturing the *Cryptosporidium*.

In an embodiment of the invention the medium employed in the culture method is a maintenance medium comprising a suitable medium for supporting protozoan such as RPMI-1640 supplemented with one or more of the following: L-glutamine, sodium bicarbonate, bile salts, glucose, folic acid, 4-aminobenzoic acid, calcium pantothenate, ascorbic acid, foetal calf serum, HEPES buffer and/or antibiotics such as penicillin G or streptomycin.

In one preferred embodiment of the invention, the culture medium is supplemented with bile. In a preferred embodiment, the concentration of bile is about 0.2 g L^{-1}.

In another embodiment of the invention, the culture medium is supplemented with an antibiotic or antifungal agent. In a preferred embodiment, the culture medium further comprises as least one additional supplement selected from the group consisting of folic acid, 4-aminobenzoic acid, calcium pantothenate and ascorbic acid.

In a further preferred form of the invention the maintenance medium will include each of the supplements mentioned above and the pH of the medium will be adjusted to a pH of 7.4. An example of the maintenance medium of the invention is formed from 100 ml RPMI-1640 (Sigma, St Louis, MI) supplemented with 0.03 g

l-glutamine, 0.3 g sodium bicarbonate, 0.02 g bovine bile, 0.l g glucose, 25 µg folic acid, 100 µg 4-aminobenzoic acid, 50 µg calcium pantothenate, 875 µg ascorbic acid, 1% FCS, 15 mM HEPES buffer, 10,000 U penicillin G and 0.0l g streptomycin, adjusted to pH 7.4.

The liquid maintenance medium may be further supplemented with an antibiotic or antifungal agent.

In an alternate embodiment of the invention the medium employed in the culture method is a biphasic medium comprising maintenance media and coagulated serum.

Preferably the coagulated serum and maintenance medium are prepared in a substantially biphasic arrangement, although one skilled in the art will appreciate that the division between the phases need not be entirely precise.

In a more preferred embodiment of the invention the biphasic media for *Cryptosporidium* development *in vitro* is generated by coagulating newborn animal (eg calf) serum. The coagulated base is then overlaid with maintenance media. Even more preferably, newborn animal serum is coagulated by incubation. This may be achieved for example by placing the serum in a water bath for 45 minutes at 70-80 ^{0}C.

In an embodiment of the culture method, step (b) is performed under conditions suitable for at least maintaining the *Cryptosporidium* in a viable state. Preferably, the conditions are suitable for promoting *Cryptosporidium* growth.

In another embodiment of the culture method, *Cryptosporidium* oocysts are introduced into the culture media. In an even more preferred form of the invention the oocysts are treated to excyst the sporozoites before being introduced into the culture media.

In another embodiment of the invention, isolated oocysts for propagation in culture are excysted by pretreatment with trypsin solution at low pH. In a preferred embodiment, the pretreatment solution comprises about 0.5% trypsin and the pH of the pretreatment solution is within a range of about 2.5 to 3. In a most

preferred embodiment, the oocysts are incubated in the pretreatment solution for about 20 minutes at about 37 ^{0}C.

In a highly preferred embodiment of the invention, the culture method comprises the steps of:

(a) isolating *Cryptosporidium* oocysts;
(b) excysting the isolated oocysts;
(c) recovering the excysted oocysts;
(d) resuspending the recovered oocysts in maintenance media;
(e) incubating the culture prepared in step (d); and
(f) recovering the oocysts.

In yet another highly preferred embodiment of the invention, the culture method comprises the steps of:

(a) isolating *Cryptosporidium* oocysts;
(b) excysting the isolated oocysts;
(c) recovering the excysted oocysts;
(d) resuspending the recovered oocysts in biphasic media;
(e) incubating the culture prepared in step (d); and
(f) recovering the oocysts.

The advantage of these culture methods is that the cryptosporidia are maintained in a host-cell free environment. This allows long-term propagation of *Cryptosporidium in vitro* and further allows development of all stages of the *Cryptosporidium* life cycle.

In one embodiment of the invention, the *Cryptosporidium* for propagation in culture are obtained from a host organism selected from the group comprising a mouse, a human, a bovine, or a pig.

In another embodiment of the invention, the *Cryptosporidium* is selected from the group comprising *Cryptosporidium andersoni* and *Cryptosporidium parvum*.

In a second aspect of the invention there is provided a culture medium for culturing *Cryptosporidium*, said media being a biphasic medium as herein described. In preparation of the biphasic medium a semi-solid media base is first

prepared from the coagulated serum phase over which maintenance medium is overlaid.

The invention also includes oocysts produced by the new culture methods described herein and compositions thereof. In a preferred embodiment, the invention includes a composition comprising oocysts produced by the new culture method and a pharmaceutical carrier.

This culturing system will be of great value for drug evaluation studies where compounds can be tested against specific *Cryptosporidium* life cycle stages.

This method also has the potential to be used for routine water and other environmental monitoring. Earlier methods of detecting *Cryptosporidium* oocysts in water samples relied on microscopic examination, although, a new cell culture system followed by PCR detection was recently developed [Rochelle *et al* (1996) J Eukaryotic Micro 43:72s; Di-Giovanni *et al* (1999) Appl Environ Micro 65:3427-3432]. However, culturing *Cryptosporidium* in host cells is labour intensive and is influenced by many factors such as host cell type and age, pH, culturing conditions (temperature, CO_2) and media supplements which can affect the parasite penetration and development. In addition, the detection of low numbers of oocysts infecting a cell culture using PCR may be subject to false positives due to the survival of nucleic acid from the inoculum in the cell culture. False positives may also occur where antigen detection methods, such as antibody-based protocols, are used. Using a cell free culture method, water samples can be concentrated by centrifugation or any other suitable system in the art, the pellet treated for excystation and then incubated in culture medium for several days. Life cycle stages that clearly represent *in vitro* development can be harvested and enumerated by any suitable system to represent the viable portion of the inoculum.

The axenic *in vitro* cultivation of *Cryptosporidium* devoid of host cells also allows the production of pure parasite material for future therapeutic development. *Cryptosporidium* extracellular stages such as trophozoites, merozoites and other extracellular gamont-like stages can be purified and surface molecules on these

stages assessed as candidate antigens for the development of passive immunotherapy agents or vaccines.

In the present invention, the terms "therapeutic" and "therapy" are used interchangeably and include, without limitation, the range of outcomes from prevention of disease, through maintenance of existing health levels to treatment of conditions and the curing of disease. The terms further include, without limitation, prophylaxis, alleviation of symptoms and restoration of health.

These additional extracellular forms of the parasite will be useful in producing a more protective therapeutic because they are likely to display antigens that are not found on intracellular forms of *Cryptosporidium.* As discussed above, extracellular forms of *Cryptosporidium* are likely to be particularly useful in eliciting an IgA response in animals. An IgA response is associated with immunity and clearing of the parasite.

The invention therefore encompasses a method of preparing a *Cryptosporidium* therapeutic by culturing *Cryptosporidium* in an appropriate *Cryptosporidium* maintenance medium in the absence of host cells. An oocyst inoculum of about 2,000 to about 5,000 oocysts/cm^2 is used in this method. Culture maintenance medium is diligently maintained at a pH between about 7.2 and about 7.6 by the addition to HEPES buffer, pH about 7.4, to the medium and by changing the medium when the pH falls outside the specified range. In one example, 15 mM HEPES is added to the medium which is replaced every 2-3 days [Hijjawi, N.S. *et al* (2001) Int J Parasitol 31:1048-55].

Medium is collected from inoculated flasks after 5 days. Oocysts and other parasite forms are recovered by centrifugation at 2000 x g for 5 minutes and the pellet is resuspended in about 2 ml PBS for subsequent use.

Alternatively, parasite-containing medium can be used to infect fresh cells or cell free media, thereby perpetuating the life cycle of *Cryptosporidium in vitro*.

The invention further provides a method of cryopreserving *Cryptosporidium* propagated by the improved culture method.

The invention also provides therapeutic compositions comprising a *Cryptosporidium* cultured as described herein which is effectively immunogenic in animals. Various strains of *Cryptosporidium* may be useful in such therapeutic compositions.

The *Cryptosporidium* may be cultured as set forth in the *Examples* section, below, and then harvested for use in therapeutic compositions. The protozoa may be disrupted prior to use in therapeutic compositions. Various methods of disruption may be used, including but not limited to sonication, osmotic pressure, freezing, exposure to detergents such as sodium dodecyl sulfate (SDS), and heating. In a preferred embodiment, sonication is used to disrupt the parasites.

In addition to disrupting the protozoa, it may be also desirable to inactivate *Cryptosporidium,* or antigens produced by *Cryptosporidium,* before use in therapeutic compositions. Conventional techniques such as heat treatment or formalin inactivation may be used.

Therapeutic compositions may comprise one or more strains of *Cryptosporidium* and/or one or more antigens of *Cryptosporidium.* Such antigens may be used in addition to whole or sonicated protozoa or may be used in cell-free therapeutic compositions.

The formulation of therapeutic compositions may include suitable pharmaceutical carriers, including adjuvants. The use of an adjuvant, for example, an alum-based adjuvant, such as aluminium hydroxide, is preferred. Commercially available adjuvants may also be used in therapeutic composition or combined with commonly available adjuvant in therapeutic compositions. For example, a preferred therapeutic composition comprises aluminium hydroxide and QUILL A (Super Fos, Copenhagen, Denmark). The precise adjuvant formulation of the therapeutic compositions will depend on the particular strain of *Cryptosporidium*, the species to be immunized, and the route of immunization. Therapeutic composition formulation is well-known to those skilled in the art.

Such therapeutic compositions are useful for immunizing an animal susceptible to *Cryptosporidium* infection, including but not limited to, bovine, ovine, caprine,

equine, leporine, porcine, canine, feline, and avian species. Both domestic and wild animals may be immunized as well as food producing animals and humans.

The present invention further provides a method of preventing or treating a disease associated with *Cryptosporidium* infection comprising administering an effective amount of a strain of *Cryptosporidium* to an animal in need of such prevention or treatment. An appropriate strain may be used in a therapeutic composition as previously discussed. This method is useful in, for example, dogs, cats, sheep, humans, domestic animals (especially food producing animals), avian species, and wild animals. Use in wild animals may prevent contamination of the environment, including water supplies used by humans or domestic animals.

Any convenient route of inoculation may be used to deliver the therapeutic composition and the route may vary depending on the animal to be treated, and other factors. Parenteral administration, such as subcutaneous, intramuscular, or intravenous administration, is preferred. Subcutaneous administration is most preferred for canine and feline species. Oral administration may also be used, including oral dosage forms which are enteric coated.

The schedule of administration may vary depending on the animal to be treated. Animals may receive a single dose, or may receive a booster dose or doses. Annual boosters may be used for continued protection. The age of the animal to be treated may also affect the route and schedule of administration. Administration is preferred at the age when maternal antibodies are no longer present and the animal is immunologically competent. These conditions occur at about 6 to 7 weeks of age in canine or feline species. Additionally, immunization of mothers to prevent infection of their offspring through passive transfer of antibodies in their milk is also contemplated. Treatment may be administered to symptomatic or asymptomatic animals, including animals or humans with chronic infection, and may be used to increase growth rate by alleviating such symptoms of infection as diarrhoea. Accordingly, administration of an effective amount of a therapeutic composition may increase feed conversion.

The present invention also provide antibodies to antigens of *Cryptosporidium*. These antibodies may be useful as an antiserum to neutralize antigens of *Cryptosporidium*, thereby relieving symptoms associated with these antigens. It is expected that oral administration of these antibodies, using an enteric coated dosage formulation, will be preferred.

The invention further provides *Cryptosporidium* that have been propagated in culture and cryopreserved. Methods of crypreservation are described in the *Examples* section below.

Best Mode(s) for Carrying Out the Invention

The following method serves to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these methods in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

Example 1: Culture preparation of *Cryptosporidium*

Maintenance Media

Maintenance medium for *Cryptosporidium* culture *in vitro* was formed from 100 ml RPMI-1640 (Sigma, St Louis, MI) supplemented with 0.03 g L-glutamine, 0.3 g sodium bicarbonate, 0.02 g bovine bile, 0.1 g glucose, 25 µg folic acid, 100 µg 4-aminobenzoic acid, 50 µg calcium pantothenate, 875 µg ascorbic acid, 1% FCS, 15 mM HEPES buffer, 10,000 U penicillin G and 0.0l g streptomycin, adjusted to pH 7.4.

Biphasic culture media

A biphasic medium for *Cryptosporidium* development *in vitro* was generated by coagulating 5-10 ml new born calf serum in 25cm^2 culture flasks for 45 minutes in a water bath at 70-80 ^{0}C. The coagulated base was then overlaid with 80 ml maintenance media.

Excystation of oocysts and culture media preparation

The *Cryptosporidium parvum* cattle genotype (Swiss cattle C26) used was originally obtained from the Institute of Parasitology, Zurich and has been subsequently passaged through mice and purified as previously described by Meloni, B.P. and R.C.A. Thompson (1996) J Parasitol 82:757-762. Oocysts used for experiments were stored in PBS and antibiotics at 5 ^{0}C before use.

Cryptosporidium parvum oocysts were excysted in freshly prepared, filter-sterilised (0.22µm filter) excystation medium composed of acidic H_2O (pH 2.5-3) containing 0.5% trypsin and incubated in a water bath at 37 ^{0}C for 20 minutes with mixing every 5 min. Thereafter, the excystation suspension was centrifuged at 2,000xg for 4 minutes at room temperature. Oocysts were resuspended in maintenance medium.

Oocyst incubation and examination of development

Flasks (25 cm^2) containing maintenance media (monophasic culture) or biphasic culture media were inoculated with 1 million excysted oocysts, resuspended in 20ml maintenance medium. The cultures were then incubated at 37^{0}C in a 5% CO_2 incubator.

Aliquots (10 ml) were taken from the flasks, centrifuged and the pellet examined for *Cryptosporidium* stages after 1, 3, 4, 8, 9, 17, 20 and 48 days of incubation. Wet mounts were prepared from the pellet and examined using Nomarski phase-contrast microscopy (Olympus BX50) and Optimas image analysis (MS-DOS operating system) for capturing images of *Cryptosporidium parvum*. Photographs were taken at x 400 and x 1, 000 magnification.

Infectivity of culture-derived oocysts to mice

Samples of maintenance medium (monophasic culture) from two 25cm^2-culture flasks containing parasites were collected from 46 day-old cultures that had been infected with 2 million oocysts of *Cryptosporidium parvum* (cattle genotype) purified from mice as described by Meloni, B.P. and R.C.A. Thompson (1996) J Parasitol 82:757-762. No media change was done to the sample through out the

culturing period. The culture medium was centrifuged at 2,000 g for 8 min and the pellet reconstituted in 2 ml PBS before being inoculated intragastrically into 7-8 day-old ARC/Swiss mice (100 µl/mouse). Eight days post-infection, the mice were processed for oocysts purification as described by Meloni, B.P. and R.C.A. Thompson (1996) J Parasitol 82:757-762.

Oocysts collected from the 46 day-old cultures were infective to 7-8 day old ARC/Swiss mice. A yield of approximately $5x10^6$ oocysts (pooled collection from 11 mice) was obtained after purification.

Excystation of oocysts and examination of development

Examination of monophasic and biphasic *Cryptosporidium parvum* cultures after 24 hrs revealed most oocysts had excysted (Fig 1d) and the presence of large numbers of sporozoites. Many sporozoites had transformed into circular to spindle-shaped motile trophozoites measuring 2x1.3µm in size (Fig 1a, b, c). Trophozoites appeared to fuse into aggregates of two or more trophozoites and occasionally large aggregates containing 10-20 stages (Fig 1a, b, and c). Between 48 and 72 hrs, trophozoites within aggregates developed into meronts (meront I) of variable size depending on the number of initially fused trophozoites (Fig 2). Meront development occurred as a result of multiple mitotic divisions of the fused trophozoites.

Consistent with our previous studies [Hijjawi, N.S. *et al* (2002) Int J Parasitol 32:1719-1726; Hijjawi, N.S. *et al* (2001) Int J Parasitol 31:1048-1055], two different types of meronts were observed (meront I and meront II) (Fig 2 & Fig 3). Type I meronts appeared as grape-like aggregates as early as 48 hrs after the start of the method of *Cryptosporidium* culturing (Fig 2). Merozoites released from these meronts were actively motile, circular to oval in shape and small in size (1.2x1µm). Merozoites released from type I meronts, enlarged and clumped together to generate type II meronts. Type II meronts, which attained a rosette-like pattern, were first detected after 3 days of culturing (Fig 3). Merozoites released from type II meronts are either broadly spindle-shaped with pointed ends measuring 3.5x2 µm in size (Fig 3b, d), or rounded to pleomorphic measuring 1.6x1.5µm in size (Fig 3c). After 7-8 days of culturing large numbers of actively

moving merozoites continued to be released from meronts (Fig 4) and from 9 days up to 46 days all developmental stages (sporozoites, trophozoites, merozoites, type I and II meronts and sporulated oocysts) were repeatedly observed in culture.

As with previous studies [Hijjawi, N.S. *et al* (2002) Int J Parasitol 32:1719-1726; Hijjawi, N.S. *et al* (2001) Int J Parasitol 31:1048-1055], it appeared that merozoites released from type II meronts, developed into the sexual stages in the *Cryptosporidium* life cycle by transforming into macrogamonts and microgamonts (Fig 5). In the present study, after 6 days of culturing (in biphasic medium), some merozoites released from type II meronts increased in size and developed into microgamonts (Fig 5a-c). Microgamonts were 5.6x5 μm in size, circular in shape and appeared very dark at low magnification (Fig 5a). The budding of developing microgametes from the surface of the microgamont stage were evident at 6 days of culturing (Fig 5a). At higher magnifications, microgametes can be easily differentiated from other stages by having a large number of developing microgametes on its surface. Microgametes were observed leaving the microgamont where an opening which resembles the suture was formed at the surface and clumps of microgametes can be detected moving freely (Fig 5d) and fully developed microgametes, measuring 2.2x1.6μm in size, were detected after 7 days of culturing (Fig 5e). Stages representing macrogamonts with characteristic peripheral nuclei were observed after 5 days and measured 5x4 μm in size (Fig 5f&g). On several occasions microgametes were observed adhering to the surface of macrogamonts and some of them were seen inside a macrogamont (Fig 5h) and sometimes a microgamete pairing with a macrogamont was also observed (Fig 5i). Stages resembling zygotes were also observed after 7-8 days and had the appearance of unsporulated oocysts with a big nucleus and measuring 5x4 μm (Fig 5j).

A significant increase in sporulated oocysts was observed after 21 days cultivation (Fig 6). The presence of oocysts at different stages of sporulation and the release of sporozoites from oocysts is evidence of the perpetuation of the *Cryptosporidium* life cycle *in vitro* (Fig 6).

Upon comparing the culture of *Cryptosporidium* in monophasic and biphasic medium two important differences were noted. First, a larger number of Meronts (I and II) were seen developing in biphasic medium, which appeared bigger in size and contained large numbers of developing merozoites than the meronts observed in monophasic medium. Second, the presence of gamont-like extracellular stages was not observed in monophasic medium but could be detected after 72 hrs of cultivation in biphasic medium (Fig 7). The extracellular stages were similar to those described by us previously [Hijjawi, N.S. *et al* (2002) Int J Parasitol 32:1719-1726]. Their size was initially small (5.3x2.3 μm) and increased with time (16.6x7.6μm) (Fig 7 a-c).

Fig 8 shows how the life cycle of *Cryptosporidium parvum* proceeded in host cell free medium and since some images are not clear, a diagrammatic representation is followed in Fig 9.

The present study has shown for the first time that *Cryptosporidium parvum*, unlike any other coccidian, can complete its life cycle without the need for host cells. This result has significant implications for the biology and classification of this parasite. For example, the present study further supports a closer affinity of *Cryptosporidium* to the gregarines than to coccidia in its development. The behaviour of *Cryptosporidium* sporozoites, once they are released from oocysts and transform into trophozoites and aggregate, leading to two merogony stages with merozoites from meront II initiating the sexual stage in the life cycle is similar to developmental stages occurring in the life cycle of the gregarine *Mattesia dispora*.

Cryptosporidium proceeds in its life cycle in the presence or absence of host cells, with developmental phases including merogony, gametogony, sporogony as well as gamont-like extracellular stages. A microgamont-like stage appeared after 6 days of culturing (Fig 5b,c), and is similar to a *Cryptosporidium baileyi* microgamont described by Cheadle, M.A. *et al* (1999) J Parasitol 85:609-615. Similarities between the two stages include the circular shape and the presence of developing microgametes, which appeared as dots, which occasionally were seen to bud off from the residuum (Fig 5b). Free microgametes were also observed after 7 days during the present study and again they appear similar to

Cryptosporidium baileyi where the nucleus occupies most of the cytoplasm (Fig 5d). The presence of sporulated oocysts after 7-8 days of culturing during the present study confirms successful fertilization.

Thus the present system provides an easy way of culturing *Cryptosporidium* without host cell interference. This culturing system is productive in terms of oocysts yield. After infecting mice with an initial inoculum of 1 million oocysts derived from the culture method described in this document, a yield of 5 million oocysts was obtained from the mice, which is indicative that viable, infectious oocysts were amplified through the *in vitro* cell free culture method. No other *in vitro* culturing system can produce such a yield.

Example 2. Cryopreservation of *Cryptosporidium* oocysts produced in culture

Cryptosporidium oocysts propagated by the methods described herein may be cryopreserved for use in future experiments, to preserve unusual parasite variants, or simply for the convenience of the practitioner. Oocysts are separated from PBS (or other resuspension medium used following harvesting) by centrifugation then resuspended in a solution suitable for cryopreservation. Cryopreservation solutions may comprise culture media, FBS, and a cryopreservant such as dimethyl sulfoxide (DMSO) or glycerol. A typical cryopreservation solution comprises 5-15% DMSO added to cell culture media comprising 10-20% FBS. Resuspended oocysts are placed on ice for several minutes, then at approximately -80°C for 2 to 3 hours, and then stored in liquid nitrogen. In an alternative method, the resuspended oocysts are placed directly in liquid nitrogen or in a cell freezing apparatus designed to control the freezing process.

Example 3. Preparing whole sonicated vaccine

A whole sonicate vaccine of *Cryptosporidium* may be prepared using, for example, a Virsonic Cell Disrupter while maintaining the cell-culture derived parasite suspension on ice. Three 20-second bursts are generally sufficient to disrupt the parasites. The presence of intact trophozoites may be checked using

a hemacytometer and an additional 20-second burst is used where necessary. The final protein concentration of the sonicate is determined using the BIORAD Protein Assay and adjusted to 0.75 mg/ml by the addition of sterile PBS. This solution is then mixed 1:4 with the previously described alum-based adjuvant for use as a vaccine preparation for immunizing animals in the following studies.

Example 4. Immunizing animals with *Cryptosporidium* oocysts produced in culture

Sonicated *Cryptosporidium* preparations, concentrated *Cryptosporidium* toxin, and other *Cryptosporidium*-containing preparations may be used to immunize animals against *Cryptosporidium*. Methods of immunizing animals against *Cryptosporidium* are adapted from those used to immunize against *Giardia* as described by Olson [U.S. Patent Nos. 5,512,288 and 6,153,191] with minor modifications.

For example, two groups of five calves each are immunized (Group A) or mock-immunized (Group B) by subcutaneous injection with about 0.5 ml of an above-described adjuvant and about 2.5 ml above *Cryptosporidium* preparation (Group A) or about 2.5 ml PBS (Group B). Animals may be checked for the presence of antibodies to *Cryptosporidium* antigens using an ELISA assay wherein purified *Cryptosporidium* antigen is immobilized on the ELISA plates. The presence of *Cryptosporidium* antibodies in the serum of immunized cattle indicates that a humoral immune response has produced antibodies to *Cryptosporidium* antigens in the vaccine.

Example 5. Challenging inoculated animals with *Cryptosporidium*

To determine whether these antibodies are protective against subsequent *Cryptosporidium* challenge, the immunized or mock immunized animals are challenged with *Cryptosporidium* parasites. *Cryptosporidium* parasites are introduced either orally or by direct intestinal inoculation. Typically, mice are challenged with about 10^6 oocysts and calves are infected with about 10^7 to about 10^8 oocysts [see, eg, Perryman, L.E. *et al* (1999) Vaccine 17:2142-49; Bukhari, Z.

et al (2000) Appl Envir Microbiol 66:2972-80; Sréter, T. *et al* (2000) Appl Envir Microbiol 66:735-738].

Example 6. Monitoring animals for clinical evidence of infection

Cryptosporidium-challenged animals are monitored for overt clinical signs of disease, including but not limited to soft stools, diarrhoea, weight loss, lethargy, and failure to thrive. Faecal cyst counts are also performed daily for the duration of the infection to determine where the infected animals are shedding *Cryptosporidium* oocysts. Serum samples are obtained at least weekly and at post mortem for use in ELISAs to measure IgM and IgG titers. Following euthanasia, gut samples (*e.g.* duodenum, jejunum, ileum) are taken for trophozoite counts, light microscopy, and electron microscopy. Mucosal scrapings, serum samples and bile are collected and stored frozen at about -80°C for further immunological analyses and enzymatic investigations. Reduced clinical manifestations of *Cryptosporidium* infection in immunized animals, compared with control animals that are not immunized, is evidence that the vaccine is effective in protecting immunized animals against *Cryptosporidium* infection.

Example 7. Enzyme linked immunosorbent assay (ELISA)

Animal gut mucosal homogenates are prepared essentially as described by Olson [U.S. Patent No. 6,153,191]. Tissue from the intestinal mucosa of infected animals is homogenized in 10% weight/volume 2 mM EDTA then stored at -80°C. Samples are then thawed and diluted about 1:1 with a solution comprising 2 mM EDTA and 1 mM PMSF. The mixture is dispersed and disrupted by five passes through an 18 G needle. Insoluble debris is pelleted by centrifugation at about 17,000xg for 20 minutes. Supernatants containing soluble proteins are used for ELISA immediately or stored at -80°C. Polyclonal or monoclonal antibodies that detect *Cryptosporidium* antigen are useful in the assay. All samples are assayed in duplicate [see also, Perryman, L.E. *et al* (1999) Vaccine 17:2142-49].

The detection of antibodies to *Cryptosporidium* proteins in the serum of immunized animals is evidence of a humoral immune response to the vaccine. By

using different purified *Cryptosporidium* antigens in the ELISA, one skilled in the art can also determine to which antigens the immunized host responds. These data can be correlated with the results of *Cryptosporidium* challenge assays to determine what antibodies afford protection against infection.

Example 8. Phylogenetic analysis of *Cryptosporidium* obtained from challenged animals

It may be desirable to perform genetic analysis on *Cryptosporidium* samples obtained from infected animals to determine whether *Cryptosporidium* that continue to propagate in immunized animals, if any, have accumulated genetic polymorphisms that allow the parasites to escape the host immune response. DNA is isolated from oocysts present in the faeces of infected animals, or from other *Cryptosporidium*-containing samples, using standard molecular biology techniques well known to those skilled in the art. At least five primers that are useful in distinguishing between *Cryptosporidium* species are described in Morgan, U.M. *et al* (1995) Am J Trop Med Hyg 52:559-64. Products obtained from PCR using these primers can be analysed directly by electrophoresis to determine if polymorphisms are evident. PCR products can also be sequenced using standard methods.

Modifications of the above-described modes of carrying out the various embodiments of this invention will be apparent to those skilled in the art based on the above teachings related to the disclosed invention. The above embodiments of the invention are merely exemplary and should not be construed to be in any way limiting.

The disclosure of each publication, patent, and patent application cited above is hereby incorporated by reference in its entirety.

Dated this TWENTY SIXTH day of FEBRUARY 2004.

Murdoch University
Applicant

Wray & Associates
Perth, Western Australia
Patent Attorneys for the Applicant(s)

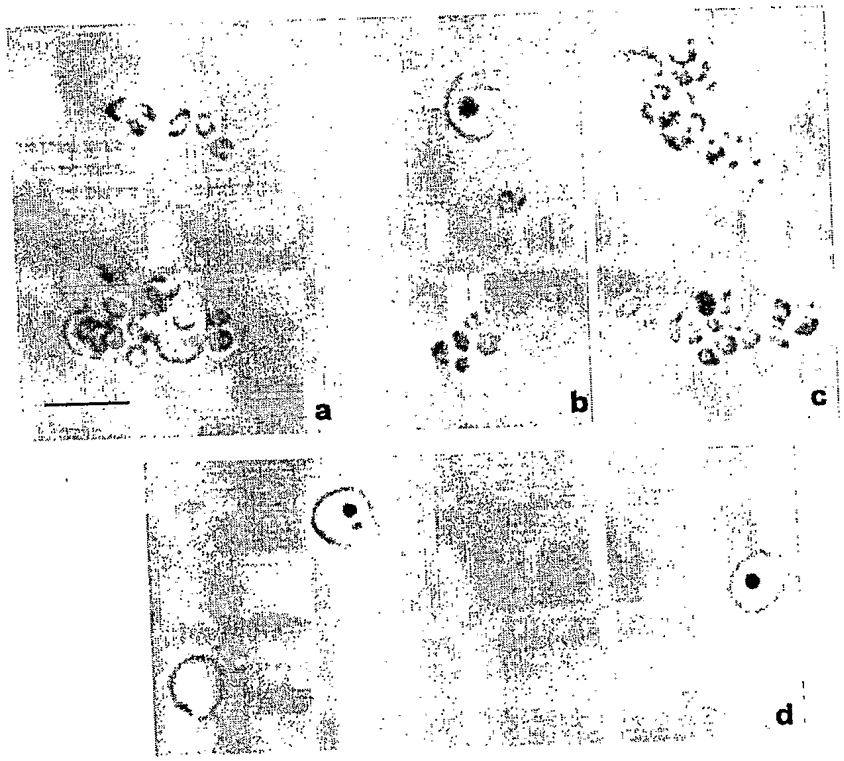
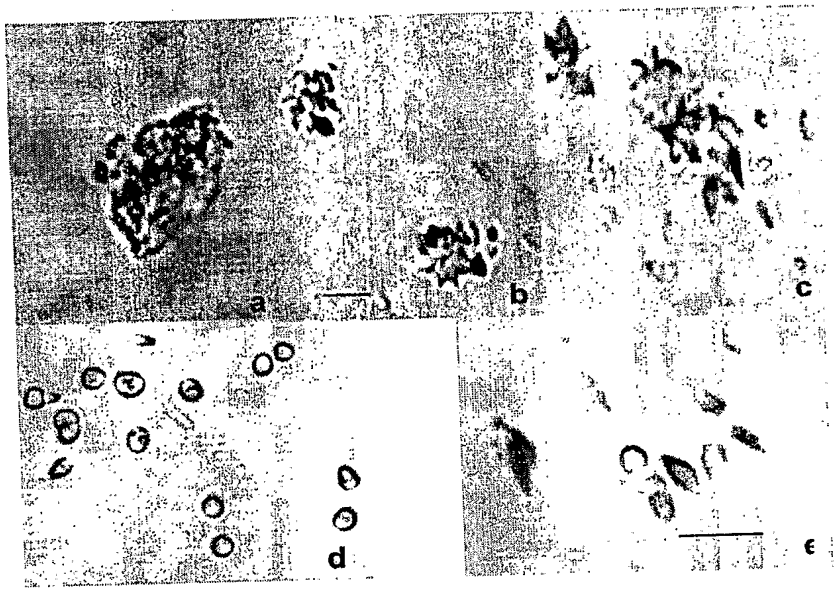


Figure 1

Figure 2



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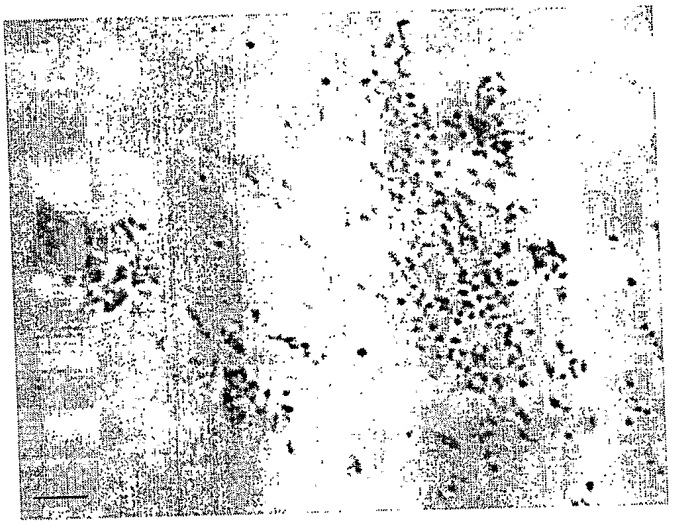

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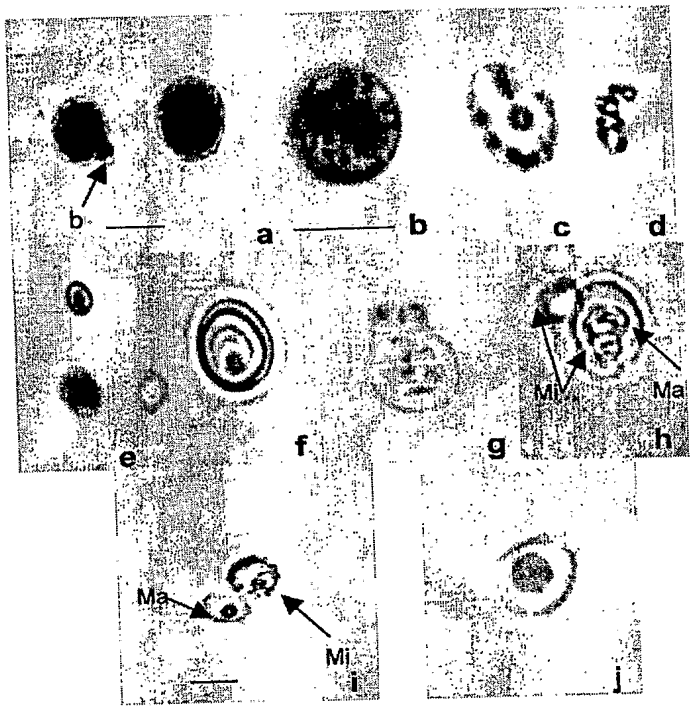

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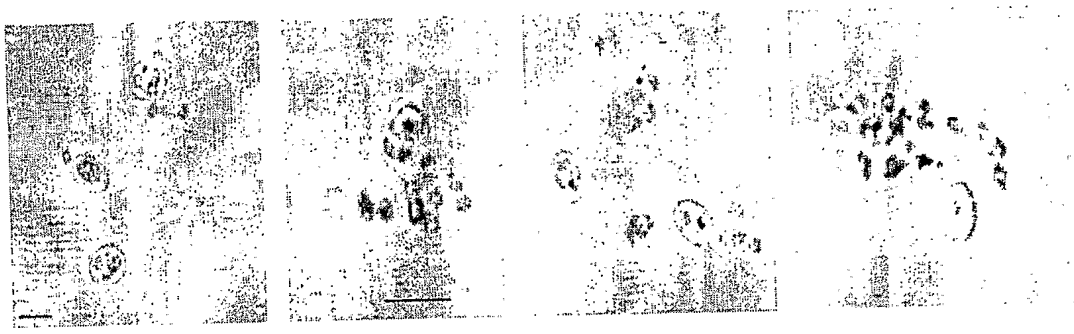

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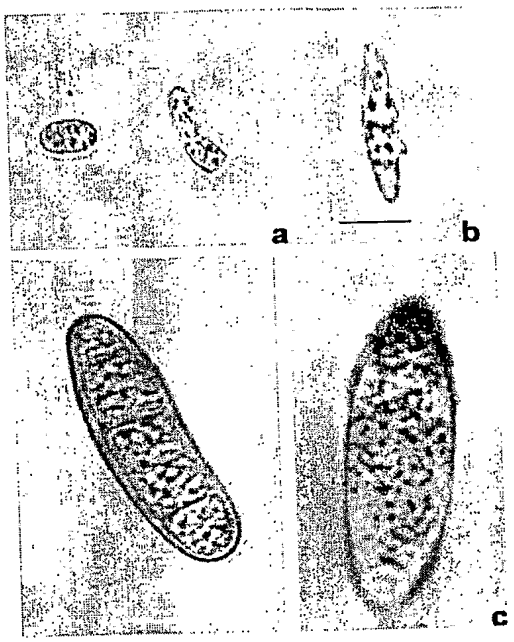

Figure 7

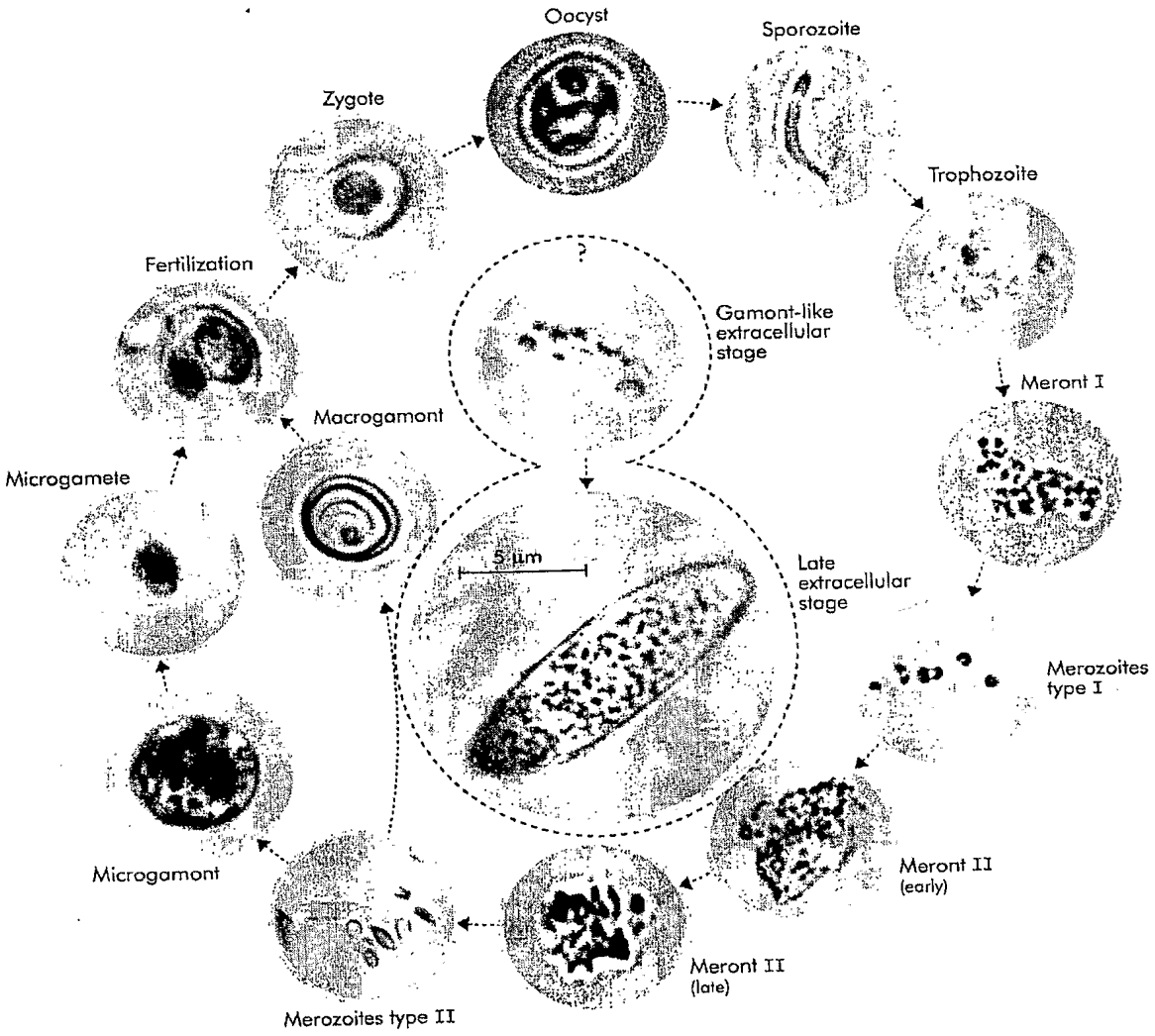
Oocyst
Sporozoite
Zygote
Trophozoite
Fertilization
Gamont-like extracellular stage
Meront I
Macrogamont
Microgamete
5 µm
Late extracellular stage
Merozoites type I
Meront II (early)
Microgamont
Meront II (late)
Merozoites type II

Figure 8

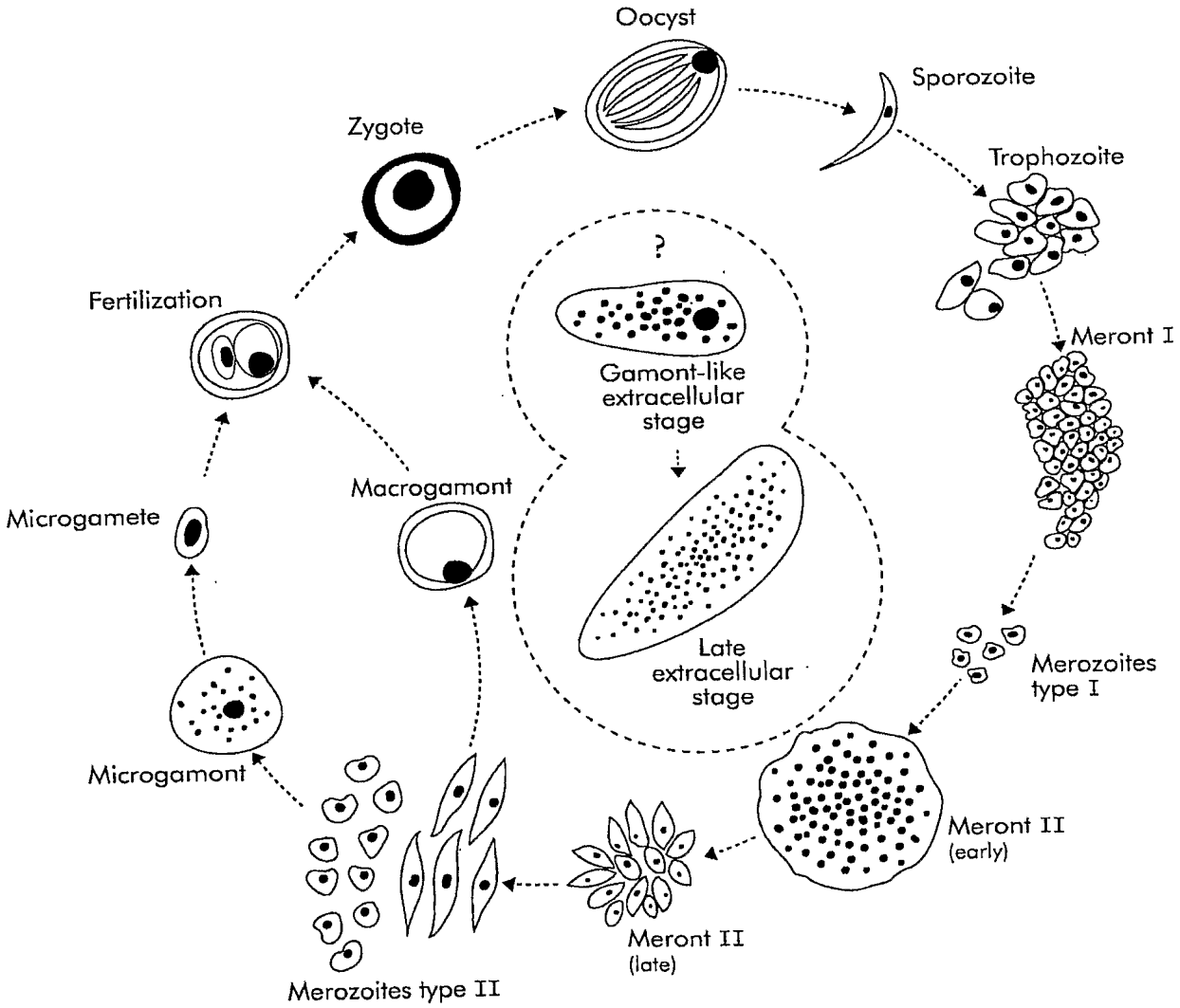
Oocyst
Sporozoite
Zygote
Trophozoite
?
Fertilization
Gamont-like
extracellular
stage
Meront I
Macrogamont
Microgamete
Late
extracellular
stage
Merozoites
type I
Microgamont
Meront II
(early)
Meront II
(late)
Merozoites type II

Figure 9